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Published in:
Human Pathology

DOI:
[10.1016/j.humpath.2013.10.003](https://doi.org/10.1016/j.humpath.2013.10.003)

2014

[Link to publication](#)

Citation for published version (APA):

Hansson, J., Hultenby, K., Cramnert, C., Pontén, F., Jansson, H., Lindgren, D., Axelson, H., & Johansson, M. (2014). Evidence for a morphologically distinct and functionally robust cell type in the proximal tubules of human kidney. *Human Pathology*, 45(2), 382-393. <https://doi.org/10.1016/j.humpath.2013.10.003>

Total number of authors:
8

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Evidence for a morphologically distinct and functionally robust cell type in the proximal tubules of human kidney.

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Running title: Cellular heterogeneity of human proximal tubules

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Key words: Progenitor cells, Acute tubular necrosis, Tubular regeneration, Mitochondria, Proximal tubules.

Funding disclosures: The authors have no connection to companies that have financial interests in the manuscript.

Conflict of interest: None

ABSTRACT

Acute tubular necrosis (ATN), elicited by ischemia and/or toxicity, is a potentially life threatening condition. Histologically, ATN corresponds to necrosis and detachment of renal tubular epithelial cells. However, the tubules possess a considerable regenerative capacity, and may be restored. We have previously identified a scattered population of progenitor like cells within the proximal tubules, sharing marker expression with the parietal epithelial cells of Bowman's capsule as well as with renal tubules regenerating after ATN. In the present analysis, we use transmission electron microscopy, immunoelectron microscopy and immunofluorescence of human kidney cortex to further explore these cells. We demonstrate that the cells are smaller and have drastically fewer mitochondria than the surrounding proximal tubule cells. They also display strong expression of several structural proteins such as vimentin, collagen-7A1 and the tight junction protein claudin-1. To functionally assess these cells, we also developed a novel human kidney explant model of ATN demonstrating that the cells are more resilient to injury than the surrounding proximal tubular cells. Taken together the results suggest a novel robust cell type with a contrasting biological role to that of the bulk proximal tubular epithelium.

INTRODUCTION

The mammalian kidney controls homeostatic core parameters such as electrolyte balance, body pH and water content, besides the more obvious task of eliminating toxins and metabolic end products. The nephrons constitute the functional units of the kidney, composed of filtering glomeruli and renal tubules, where the primary filtrate is modified and largely reabsorbed. Nephrons are cellular mosaics, enabling the tubules to be divided into histologically and functionally diverse parts. Proximal tubules performs the major part of the reabsorptive process and thus require massive amounts of energy, rendering them very susceptible to nutrient and/or oxygen loss that may, if severe, cause acute tubular necrosis (ATN). Major inroads to ATN are toxicity or ischemia, where the latter is most often due to circulatory disturbances [1]. Clinically, ATN represents the most common cause of the potentially lethal syndrome of acute renal failure [2]. ATN causes the tubular cells to lose polarization, followed by necrosis or apoptosis, cellular detachment and ultimately denuded basal membranes. Remarkably, the otherwise mitotically quiescent kidneys have a pronounced capacity for tubular cellular regeneration following damage. Which events and what cells that are responsible for this process has been debated.

The classical view is that tubular regeneration is performed by the stochastically least damaged tubular cells that are thought to dedifferentiate and divide in order to repopulate the basal membranes [3]. This view is still gathering qualified experimental support by lineage tracing and metabolic labeling experiments [4, 5]. A recent, and possibly complimentary hypothesis is that regeneration is performed by adult resident renal stem cells [6], suggested to reside in the parietal epithelial cell (PEC) layer of Bowman's capsule [7], the renal interstitium [8, 9], or the renal papilla [10]. The hypothesis that PECs indeed constitute a source for stem cells has gained substantial experimental support during recent years. For example these cells have been shown to replenish glomerular podocytes [11, 12]. It has also been speculated that PECs may take part in regenerating the initial part of the proximal tubules.

The hypothesis that there might exist a distinct cell population in human proximal tubules, that may be involved in regeneration has recently been added interesting experimental support by a number of groups, suggesting several mechanisms for their role in kidney injury [13-16]. Importantly, the tubular progenitor cells are immunophenotypically almost identical to the PEC progenitor cells [13, 14]. However, Angelotti et al. showed that tubular progenitors may

be distinguished from the Bowman's capsule progenitors by absence of the surface marker CD106. Isolated human tubular CD106^{neg} cells were able to incorporate in renal tubules of SCID mice following renal damage and generate new epithelial cells that improved the recovery of the mice [13]. A report by Sallustio et al. provided mechanistic insight into the function of the tubular progenitors by showing that renal tubular stem cells could protect renal proximal tubule cells (HK2 and RPTEC), from cisplatin induced injury in a TLR2 dependent manner via exosomal exchange [17]. This tubular cell population may be interpreted alternatively however, as recently by Smeets et al, instead describing a *de novo* occurring cell type with KIM-1 positivity that occurs first after injury in rat, to aid renal regeneration. In this report, the cells are thought to arise through dedifferentiation in alignment to the classical view on renal regeneration [18].

Using the Aldefluor stem cell assay, we have previously isolated a rare population of cells from human kidney cortex with progenitor characteristics. Immunohistochemical characterization showed that these cells were scattered through the proximal tubular epithelium [14]. The present study expands the evaluation of these cells, which we herein call proximal tubule rare cells (PTRC), and investigates their ultrastructural morphology. Our results provide evidence for the presence of specialized cells within proximal tubules, marker-wise similar to PECs, and with biological properties distinct from that of their surrounding proximal epithelium. We finally develop and use a novel human explant ATN assay to show that scattered PTRCs indeed are more prone to resist cellular injury than the surrounding bulk cells.

MATERIAL AND METHODS

Acquisition of renal tissue

Human renal tissue was obtained from several sources. Normal cortical tissue with minimal fibrosis and vascular disease were obtained from kidneys nephrectomized due to malignancy. This tissue was fixed immediately after nephrectomy. Tissue was also obtained from pre-transplant biopsies and from renal biopsies due to medical renal diseases. All samples were collected after informed consent by the patient. Ethical permission from the ethical committee at Lund University (LU680-08 and LU 289-07).

Transmission Electron Microscopy (TEM)

Human renal tissue was fixed in 2 % Glutaraldehyde buffered with 0.1 M cacodylate/0.1 M sucrose buffer at pH 7.2. Following 60 minutes osmification and dehydration tissue was embedded in Agar 100 Resin and cured for 2 days at 60 °C. Ultra thin sections were subsequently stained with 2 % uranyl acetate for 25 min and lead citrate for 2 min, and viewed using a Philips CM 10 electron microscope.

Immunoelectron microscopy (iEM)

Normal kidney was fixed in 3 % paraformaldehyde in 0.1 M phosphate buffer, washed in buffer and then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Sectioning was performed at -95 °C. Immunolabeling procedure was performed as described earlier [19] using vimentin antibody (ab45939; Abcam).

Immunofluorescence

Sections of 4 µm from formalin fixed and paraffin embedded kidney cortex were deparaffinized with xylene, followed by graded ethanol series. Heat mediated antigen retrieval was performed with Diva Decloaker solution (Biocare Medical). For Cryosections, 4 µm sections were fixed for 5 min in ice-cold acetone. Sections were subsequently washed in phosphate buffered saline (PBS) and blocked in 3 % bovine serum albumin (BSA) (MP Biomedicals) in phosphate buffered saline 10 % tween (PBST). Sections were incubated with primary antibodies in blocking solution over night at 8 °C and with secondary antibodies for 1h at room temperature. Applied antibodies can be found in Supplemental table 1. Sections were subsequently analyzed by confocal scanning using the Zeiss LSM 710 system (Carl Zeiss AG).

Immunohistochemistry

For COL7A1-CD133 co-staining; 4 µm paraffin sections of human kidney cortex were deparaffinized with xylene, followed by a graded ethanol series. Endogenous peroxidase was blocked with 0.3 % H₂O₂ in 95 % ethanol, 5 min. Heat mediated antigen retrieval was performed in retrieval buffer, PT module buffer pH 6®, pH 6 (TA-050-Pm1X), using a pressure boiler. Antibody staining was performed in Autostainer 480® (Thermo Fisher Scientific Anatomical Pathology, Astmoor Runcorn, UK) for 30 min at room temperature, washed (x2) and incubated with secondary bridge antibody for 30 min. Sections were washed (x2) and incubated with 1:st MACH 2 polymer (Biocare Medical, Walnut Creek, CA) for 30

min, washed (x3) and developed in Vulcan Fast Red chromogen kit (Biocare Medical Walnut Creek, CA) for 10 min. Rinsed in distilled water (x3), followed by incubation with second primary antibody for 30 min thereafter treated as above and developed in Ferangi blue chromogen kit (Biocare Medical, Walnut Creek, CA). Antibodies used: rabbit anti-CD133, 1:25 (HPA004922; Sigma Atlas antibodies), mouse anti-COL7A1 1:25 (sc-33710, Santa Cruz). The antibodies were obtained from the Human Protein Atlas project, for full profiling of these proteins and antibodies visit www.proteinatlas.org. Remaining immunohistochemistry was performed as previously described [14]. See antibody information in supplemental table 1.

Renal explants –Acute tubular necrosis ex vivo

Human renal tissue was obtained from kidneys nephrectomized due to malignancy. Nephrectomy requires surgical clamping of the renal vessels and this event was used as starting point for development of ATN since the kidney then is subjected to complete ischemia. After a period of 90 min in ischemia, pieces of human cortical tissue farthest from the malignancy was dissected out and put in cold basal medium; Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (PEST). The tissue was cut into approximately 0.4 x1.5 cm pieces and placed in tissue culture plates containing basal media and were allowed to reoxygenate during standard cell culture conditions at 37 °C in 5 % CO₂. The ATN process was allowed to develop *in vitro* up to a maximum of 72 h. The pieces were harvested at different time points 0 h, 2.5 h, 20 h, 28 h, 42 h, 50 h and 72 h, fixated in 4 % PFA and embedded in paraffin. FFPE sections of 4 µm were stained with the following antibodies: CD133, KRT7, KRT19, CLDN1, VIM, as described in the immunohistochemistry and immunofluorescence section. The frequency of cells were calculated using CD133 stained sections and counting the number of CD133⁺ versus CD133⁻ cells attached to the basal membrane in proximal tubules in the 72 h samples versus 0 h samples, as a mean of 4 images.

RESULTS

Rare proximal tubule cells similar to cells of Bowman's capsule

We have previously described a progenitor-like cell within the human renal proximal tubules [14]. The cells are characterized by co-expression of the stem cell markers CD133, CD24 and basal vimentin expression (Figure 1 A-C). They are often localized to tubular creases (Figure 1 A) and sometimes found in duplets (Figure 1 C). The proximal tubule rare cells (PTRCs) are immunophenotypically similar to the parietal epithelial cells (PEC) of Bowman's capsule and share characteristic markers like CD24, CD133 and vimentin (Figure 1 C-E).

Scattered vimentin positive cells present in proximal tubules in human kidneys of all ages

Vimentin positivity is often associated with regenerating, dividing, tubular cells after renal injury [20]. Importantly, however, the presence of scattered cells were detected in human kidneys of all ages, in the similar proportions, and were not exclusive to renal tissue from cancer patients, but also present in wedge biopsies from healthy kidneys (Figure 2).

Calculations using stained sections of kidneys from patients of varying ages (13-60 years), showed a frequency of 3-12 % of scattered vimentin positive cells within proximal tubules, with no apparent association between age and frequency.

Scattered proximal tubule cells display low mitochondrial content

To ultratructurally characterize the PTRCs we performed transmission electron microscopy (TEM) on human kidney cortex. Proximal tubule cells are normally completely filled with mitochondria. The TEM images showed the characteristic mitochondrion dense proximal tubule cells, but also a rare subset of cells scattered within the proximal tubules appearing to have drastically fewer mitochondria (Figure 3 A-C), in line with the previous report by Smeets et al. [18]. The cells also displayed a basal mat of electron dense material and a darker, seemingly more compact nucleus, not seen in surrounding proximal tubule cells. The mitochondrial-low cells sometimes appeared in duplets much like the CD133/CD24/Vim positive proximal tubule cells (Figure 3 B). To confirm that these mitochondrial-low cells indeed were identical to the scattered cells previously identified by us, immunofluorescence (IF) was employed. Human renal cortical tissue sections were co-stained for the mitochondrial marker cytochrome c oxidase II (MTCO2) and CD133. Results showed that CD133 positive cells had significantly fewer mitochondria than the surrounding proximal tubule cells (Figure 3 D). We also co-stained the mitochondrial markers MTCO2 or VDAC1 with the previous identified [14] PTRC-markers CD24, vimentin and the cytoskeletal protein

cytokeratin 19 (KRT19). Results replicated the CD133 staining where the positive cells had lower mitochondrial content (Figure 3 E-G). Additionally, we performed immunoelectron microscopy (iEM) using gold-conjugated vimentin antibodies that corroborated these findings (Figure 4). As expected, a high concentration of labeling for vimentin was found in the glomerulus, podocytes, and in the cytoplasm of endothelial cells (data not shown). In some proximal tubules, a few disperse cells showed high and specific labeling for vimentin, localized to a basal filamentous mat structure (Figure 4 A, B). Adjacent proximal tubule cells showed negligible labeling (Figure 4 C). The labeling pattern was streak-like and highly associated to the cytoplasmic filaments at the basal compartment. (Figure 4 D). In comparison to the morphology of bulk proximal tubule cells, displaying a dark cytoplasm and numerous mitochondria, the vimentin positive cells presented a light cytoplasm, few mitochondria and distinct intracellular filaments. In summary the EM and IF findings confirm the presence of a subset of structurally distinct vimentin positive, mitochondrial-low cells in proximal tubules.

Intermediate filaments and structural proteins in PTRCs

In our previous gene expression analysis [14] we noted that two other structural proteins were upregulated in the PTRCs: the anchoring protein collagen-7A1 (COL7A1) and the tight junction protein claudin-1 (CLDN1). Co-staining of renal cortex for COL7A1 and CD133, or CLDN1 and vimentin showed co-localization in scattered proximal tubule cells, (Figure 5 A-B and C respectively). We also detected COL7A1 and CLDN1 expression in PECs of Bowman's capsule, again underscoring the marker similarities to the tubular progenitor cells (Figure 5 B and D respectively). CLDN1 was also detected in distal convoluted tubules and collecting duct, in line with previous publications [21]. In co-stainings of CLDN1 and MTCO2 the CLDN1 positive cells displayed lower mitochondrial content (Figure 5 E-F). In conclusion we show that the PTRCs have drastically fewer mitochondria and an apparently more structurally robust phenotype compared to the bulk proximal tubule cells.

Human kidney explant model – ATN ex vivo

To investigate the hypothesis that the PTRCs may be more robust than the bulk epithelial cells during injury, we developed an explant model to emulate ATN *ex vivo* using nephrectomized human kidneys. In conjunction with a nephrectomy procedure the kidney developed ischemia that was allowed to continue for 90 min. Pieces of kidney cortex were thereafter reoxygenated under tissue culture conditions, and the ATN process was allowed to develop *in vitro* up to a maximum of 72h. Not surprisingly the tissue showed morphological

signs of massive ATN with partially or fully denuded basal membranes and stretches of detaching cells sloughing into the tubular lumen. (Figure 6 A-F), (Supplemental Figure 1 A-E). Importantly however, scattered cells double-positive for the PTRC-markers, CD133, KRT7, KRT19, vimentin and CLDN1 remained anchored to the basal membranes, (Supplemental Figure 1 C'-E'), whereas the detaching cells were negative for these markers. Also the positive cells were often found at the proper micro-anatomical sites in the tubules, such as tubular plicae where proximal convoluted tubules make hairpin turns (Figure 6 B-C, F). We also calculated the proportion of bulk and PTRC cells that outlasted the 72-hour hypoxia/reoxygenation experiment. About 20 % (107 +/- 45 of 667 +/- 40 cells, mean +/- SD) of the bulk proximal tubular cells outlasted the 72-hour hypoxia/reoxygenation experiment. In contrast 60 % (17 +/- 4 of 27 +/- 7 cells, mean +/- SD) of the CD133 expressing cells endured the procedure. These results further substantiate the hypothesis that PTRCs are more resilient to environmental stress occurring during ATN, than surrounding bulk epithelial cells.

PTRCs in clinical samples with diagnosed tubular injuries

To further investigate the role of PTRCs during regeneration we analyzed clinical samples from renal diseases where the tubular injuries were distinct and where the initiating event could be timed reasonably well. We sought to demonstrate three time frames, acute, subacute and chronic injury. To this end we included samples from acute tubular necrosis secondary to kidney transplant or pauci-immune glomerulonephritis (Figure 7 A-D), a prolonged case of crescentic glomerulonephritis due to delay (Figure 7 E-G) and finally a case of chronic urinary obstruction (Figure 7 H-J). Light microscopy showed that the biopsy displayed clear signs of ATN with tubular cells sloughing into the lumen, distinct reduction of epithelial height and diminished brush border. In parallel regeneration of tubules was also observed where stretches positive for the PTRC-marker CLDN1 could be seen (Figure 7A). Stretches of cells double positive for CD133 and vimentin/KRT19/KRT7 were furthermore demonstrated (Figure 7 B-D). In panel 7 E-G a more subacute ischemic injury is shown where, again, regenerating tubules are seen expressing the PTRC-markers CD133, vimentin, KRT19 and CLDN1. The final series of images shows a chronic injury due to urinary obstruction (Figure 7 H-J). Areas of fibrosis could be seen adjacent to areas of ongoing regeneration expressing the same set of markers. This indicates that the PTRCs are active even after more prolonged injuries.

DISCUSSION

In this paper we present the ultrastructural features of a histologically distinct cell type scattered through the epithelium of the proximal tubules (Figure 8). Previously we isolated and characterized this cellular heterogeneity at the level of gene expression and light microscopy [14]. These findings were supported and expanded in a recent publication by Angelotti et al. [13] and the heterogeneity of proximal tubules have since then been reported by other groups, albeit with alternative conclusions regarding the role of these cells [17, 18]. In our previous study we provided evidence for the scattered cells role as renal progenitors and presented a series of defining markers such as CD133, CD24, KRT7, KRT19 and vimentin [14]. CD133 has been used as a marker for the identification and isolation of progenitor cells in both human and murine tissues [22-24]. In the kidney, CD133 expression has been ascribed to progenitor cells of Bowman's capsule [7, 12], and to occasional cells in the renal interstitium [25], or papilla [10], while other studies have described cells with cytoplasmic and membranous CD133 positivity in most renal tubules [15, 26]. In contrast we only detect apical CD133 expression in scattered proximal tubule cells and in PECs of Bowman's capsule. This discrepancy is probably due to the specificity of different antibodies applied, specifically whether or not they recognize the glycosylated forms of the protein, as previously reported [27].

Using iEM and TEM we here present a vimentin expressing, wedge shaped and histologically distinct cell type, with a striking paucity of mitochondria. By immunofluorescence co-stainings we show that the mitochondrial-low cells indeed are identical to the PTRCs. The low mitochondrial content in scattered proximal tubule cells has recently also been reported by Smeets et al. [18]. Collectively our present and previous data [14], together with recent reports [13, 17], indicate a scattered proximal tubule cell population with a higher probability of surviving renal injury in comparison to surrounding cells due to their robust phenotype. We hypothesize that this increased cellular robustness is mediated by antiapoptotic signaling, lower oxygen requirement and increased mechanical resilience. Previous data showed that these cells demonstrate increased BCL-2 expression; contributing to antiapoptotic features [14].

The second trait of robustness may be met by the mitochondrial paucity of these cells. Mitochondrial load varies considerably between cell types in proportion to energy

requirements. High energy demand due to vectorial solute transport by proximal tubules is reflected by high mitochondrial load, rendering this epithelium extremely sensitive to oxygen/nutrient deprivation [1]. The lower mitochondrial load of PTRCs may thus increase resilience to hypoxia and grant an increased probability of survival. Combining mitochondrial paucity with BCL-2 expression may make these cells particularly resilient to apoptotic cues. A low number of mitochondria could also minimize the risk of genomic alterations due to formation of reactive oxygen species associated with mitochondrial activity [28]. Furthermore, it has recently been suggested that undifferentiated cells per definition have lower mitochondrial mass and use non-oxidative glycolysis as their major source of energy [29].

Our third postulate, regarding increased mechanical resilience, is supported by the basal vimentin expression. Vimentin contributes to stress resilience and increased rigidity upon cellular strain, and vimentin-depleted cells have been shown to be extremely vulnerable to insults [30]. Additionally, vimentin is also regarded as an anti-apoptotic factor [31] being expressed in regenerating renal tubules following ischemic injury in both human and rat [20]. In this study we also present two further markers for the renal progenitors, the structural proteins COL7A1 and CLDN1. COL7A1 is a rare form of collagen that in contrast to other collagens only serves structural properties. COL7A1 anchors the basal membrane of epithelia to the underlying extracellular matrix and is a prerequisite for epithelia with high demand for mechanical robustness, such as the epidermis of the skin where dysfunctional COL7A1 leads to the severe blistering disease epidermolysis bullosa [32]. The presence of COL7A1 in the PTRCs may denote an important attachment system, perhaps working in conjunction with vimentin and the tight junction protein CLDN1, to firmly anchor these cells to the extracellular structures.

In a recent study Smeets et al. reported that the scattered CD133/CD24 positive tubular cells co-expressed kidney injury molecule-1 (KIM-1) [18]. Additionally, they only find negligible staining of vimentin in healthy rat kidneys, while after induced renal injury they noted a clear increase of vimentin positive cells. The authors interpret these results as indicative of *de novo* occurring dedifferentiating cells, and explain the detection of the scattered cells in human samples as a consequence of renal wear and tear in the nephrectomized kidneys from older patients. Our vimentin stainings of healthy kidneys from ages 2 and up (Figure 2) would argue against this notion since we detect scattered vimentin positive cells in the similar

proportions in all samples, regardless of age. Regarding the KIM-1 positivity described by Smeets et al. it should be emphasized that KIM-1 is not necessarily only associated with dying cells. The expression of KIM-1 is indeed known to increase after injury, much like vimentin expression, but recent findings suggest that KIM-1 also plays a role in the tubular regeneration process [33]. In order for the regeneration to occur properly, dead cells and debris must be cleared away, and KIM-1 expression is thought to contribute to a phagocytic phenotype that entails a scavenger function of epithelial cells [34]. Additionally, Smeets et al. interpret the overall marker expression of the scattered proximal tubular cells as signs of dedifferentiation. Importantly though, virtually all described PTRC-markers are however shared with the PECs of Bowman's capsule; hence these markers are not exclusive to dedifferentiating cells.

There are compelling evidence from mice studies that the PECs of Bowman's capsule constitute a progenitor cell compartment able to differentiate into podocytes [11], and that human PECs injected into mice with renal injury can give rise to both podocytes and tubular cells [7, 12]. As the tubular progenitors bear a striking marker similarity to PECs, it is tempting to speculate that scattered progenitor cells in proximal tubules are required to ensure a rapid and effective regeneration upon renal injury due to the distance to Bowman's capsule in humans.

To functionally assess the robustness of the PTRCs in comparison to bulk PT-cells we developed a novel *ex-vivo* explant culture system where the vascular clamping of human kidneys undergoing nephrectomy was utilized to simulate ATN. After a defined exposure to ischemia in conjunction with nephrectomy, renal cortical tissue was allowed to reoxygenate in a cell culture environment. The procedure produced massive tubular necrosis, however leaving cells with PTRC-markers intact in the tubular cross-sections. Notwithstanding the limitations of the assay, this observation supports the underlying hypothesis that the PTRCs are more resilient to insult than bulk epithelium.

Additionally, we show that stretches of surviving tubular cells in biopsies from patients with both acute and prolonged tubular injury express PTRC-markers. These observations are compatible with a model by which the PTRCs, not only survive, but also proliferate upon renal injury, thereby providing the cellular source for regeneration. An alternative, and not necessarily mutually exclusive model, was recently suggested by Sallustio et al. [17]. In

accordance with our results of cellular robustness, they showed that purified tubular progenitor cells were more resilient to cisplatin-induced toxicity. They could also show that the tubular progenitor cells, in contrast to glomerular progenitors, could both protect renal epithelial cells from cisplatin induced cell death and induce proliferation of these cells in a TLR2 dependent manner [17].

The integrity of renal tubular epithelium is essential and it remains a possibility that there are several different systems for kidney regeneration that can be activated, dependent of the type and severity of the insult. The PTRCs may provide a last line of defense in renal injury, if the degree of tubular necrosis has been too severe for restoration by bulk epithelium alone.

ACKNOWLEDGEMENTS

Ms Elise Nilsson is acknowledged for outstanding immunohistochemistry. Dr Birendra Singh is acknowledged for the excellent artwork of the schematic image of the progenitor cell compartment. This research was supported by The Marianne & Marcus Wallenberg Foundation, the Swedish Research Council, the National Association against Kidney Diseases, Regional ALF Funds, Malmö University Hospital Research Funds, the Knut and Alice Wallenberg Foundation, Gunnar Nilsson's Cancer Foundation, the strategic Cancer Research Program Biocare and the Swedish Cancer Society.

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FIGURE LEGENDS

Figure 1. Scattered divergent cells in human renal proximal tubules share marker phenotype with Bowman's capsule. Immunofluorescence staining of renal cortex. (A) CD133 & vimentin co-staining showing a proximal tubule (PT) with a single cell at a plicae position with apical CD133 and basal vimentin (arrow). Vimentin positivity is also seen in surrounding interstitial cells. (B) Cryosection stained with vimentin and CD24 showing double positive single cell (arrow) vimentin positivity is also seen in surrounding interstitial cells. (C) CD133/CD24 co-staining of a cryosection showing a double positive cell duplet (arrow) in a proximal tubule. Co-positivity is also seen in Bowman's capsule surrounding a glomerulus (G). (D) CD133/vimentin co-staining shows positivity in Bowman's capsule. (E) CD24/vimentin co-staining show double positive cells in Bowman's capsule. Arrows indicate areas of interest shown at a higher magnification, and in separate fluorochrome channels beside the merged images. Scale bars (A, C-E): 50 μm , B: 20 μm .

Figure 2. Scattered vimentin positive cells present in proximal tubules in healthy kidneys of all ages. Immunohistochemical stainings of pre-transplant biopsies from native donors. (A-E) Co-staining of vimentin (brown) and the proximal tubule marker CD10 (red). (A) 50 year old donor. (B) 20 year old donor. (C) 13 year old donor. (D) 13 year old donor. (E) 12 year old donor. (F) Vimentin staining of a biopsy from a 2 year old donor. Arrows points to scattered vimentin positive cells.

Figure 3. Proximal tubule rare cells display low mitochondrial content. (A-C) Transmission electron microscopy images of proximal tubules (PT). (A) Overview of a proximal tubule (PT) showing a divergent, small, flask shaped cell with sparse cytoplasm, dark nucleus (N) and few mitochondria (arrow). (B) Overview of proximal tubule showing a divergent small cell duplet (arrow). (C) Higher magnification of B, the duplet is resting on a filamentous mat (arrowhead), display condensed chromatin and a dramatic paucity of mitochondria, (Mi) compared with the cytoplasm of the adjacent cell. (D-G) Immunofluorescent co-staining of mitochondrial and PTRC-markers in human renal cortex. (D) Co-staining of CD133 and MTCO2, show CD133 positivity in Bowman's capsule and in a cell duplet in PTs. CD133 positive cells display absent mitochondrial staining. (E) Staining with CD24 and mitochondrial marker MTCO2 on a cryosection show PTs with CD24 positive cells with absent MTCO2 staining. (F) Staining with vimentin and mitochondrial marker MTCO2 show PTs with vimentin positive cells with absent MTCO2 staining. Interstitial cells

also display vimentin positivity. (G) Mitochondrial marker VDAC1 and KRT19 staining show single KRT19 positive cell with low VDAC1 intensity in PTs, as well as the expected KRT19 positivity in distal tubules (DT). Arrows indicate areas of interest shown at a higher magnification, and in separate fluorochrome channels, to the right of the merged images. Scale bars: (A-B) 8 μm , (C) 4 μm , (D-F) 50 μm , (G) 100 μm .

Figure 4. Immunoelectron microscopy reveal vimentin positive cells with low mitochondrial content and abundant intermediate filaments. (A) Overview of proximal tubule showing a cell with a light cytoplasm at the border of the basement membrane (BM) surrounded by tubule cells (TC). (B) Higher magnification of the cell in A demonstrating a high and specific vimentin labeling in the cytoplasm by gold coupled secondary antibodies. N = nucleus, Mi = mitochondria. (C) Single cell with high vimentin labeling compared to the negligible labeling in the adjacent tubule cells (TC), arrowheads delineate cell border. (D) Vimentin labeling displays a streak like a pattern and is highly associated to the filaments (arrowhead) seen in the cytoplasm. Scale bars: (A) 5 μm , (B, C, D) 500 nm.

Figure 5. Proximal tubule rare cells are positive for COL7A1 and the tight junction protein CLDN1. (A, B) Immunohistochemical staining of the structural protein COL7A1 and progenitor marker CD133, show co-localization in scattered proximal tubule cells (arrow). (C) IF staining for the tight junction protein CLDN1 and vimentin show co-localization in single cells in proximal tubules in typical plicae position. As well as the expected CLDN1 positivity in collecting ducts (CD). (D) IF staining of CLDN1 and vimentin, show co-localization in parietal epithelial cells of Bowman's capsule. (E-F) Co-staining with CLDN1 and MTCO2 show scattered CLDN1 positive cells with lower mitochondrial content. Scale bars: (A) 200 μm , (B-F) 50 μm .

Figure 6. Cells with PTRC-markers are resilient to ATN-like insults. (A-F) Staining of renal cortical issue after 72h of reoxygenation following clamping of kidney. (A) Overview of kidney cortex, H & E staining. (B) Immunohistochemical staining of vimentin. (C) Immunohistochemical staining of CD133. (D) Immunohistochemical staining of CLDN1. (E) Immunohistochemical staining of KRT19. (F) Immunohistochemical staining of KRT7. Arrows show cells positive for the progenitor markers attached to the basal membrane. G= glomerulus, CD= collecting duct. Scale bars: (A) 100 μm , (B-F) 50 μm .

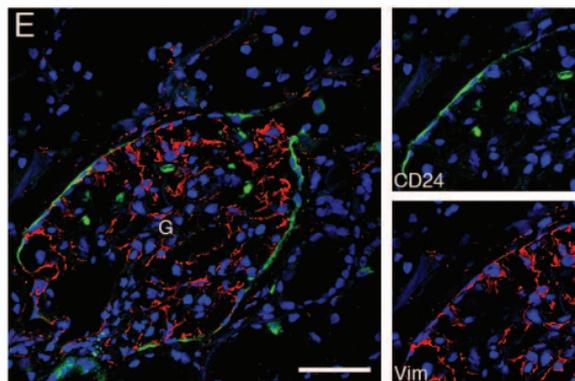
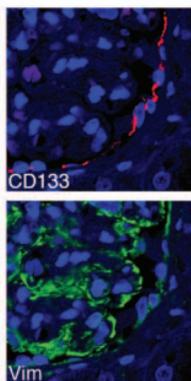
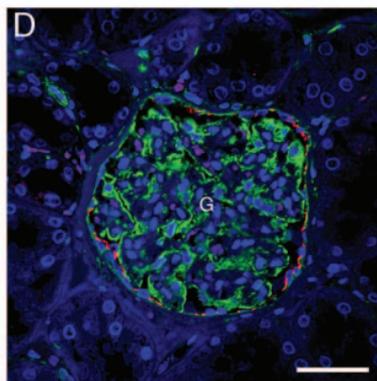
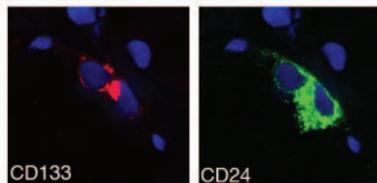
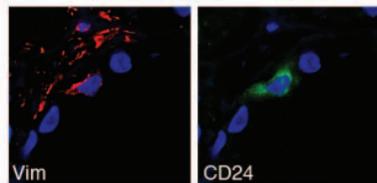
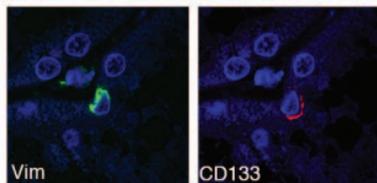
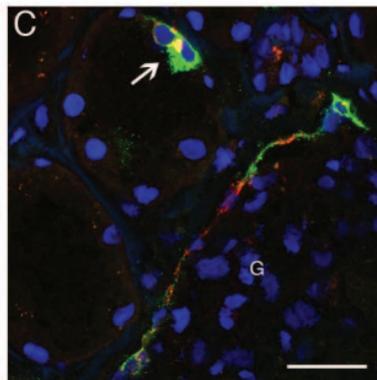
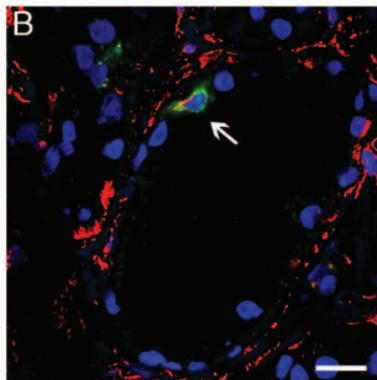
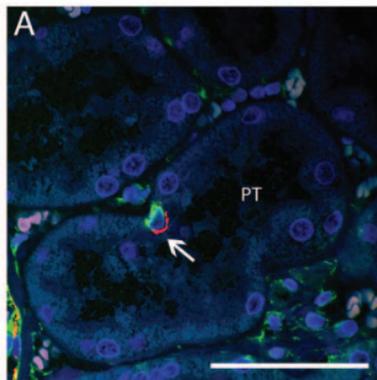
Figure 7. PTRCs in primary and secondary tubular injuries. (A-D) Images from biopsies where clear signs of acute tubular necrosis could be seen using light microscopy. The biopsies were taken from a kidney transplant and pauci-immune glomerulonephritis where ischemia was initiated about 3-4 days previously. (A) A stretch of CLDN1 positive cells with ample cytoplasm is seen in a tubular cross-section also showing signs of necrosis and reduced cellular height in injured cells where the latter show no expression of CLDN1. Immunohistochemistry has been used to show the tissue structure more clearly. (B-D) CD133 is co-stained with, vimentin, KRT19 and KRT7 respectively, showing the same pattern. (E-G) Renal biopsy from a case of crescentic glomerulonephritis where the ischemic insult was more subacute-chronic and dated due to treatment delay. Also here regenerating tubules can be seen demonstrating generalized expression for the PTRC-markers CD133, vimentin, KRT19 and CLDN1. (H-J) Renal biopsy from a case of chronic urethral obstruction display fibrotic areas intermingled with areas of PTRC-marker-positive regenerating tubules. Arrows indicate areas of interest shown at a higher magnification, and in separate fluorochrome channels beside the merged images. Scale bars: 50 μ m

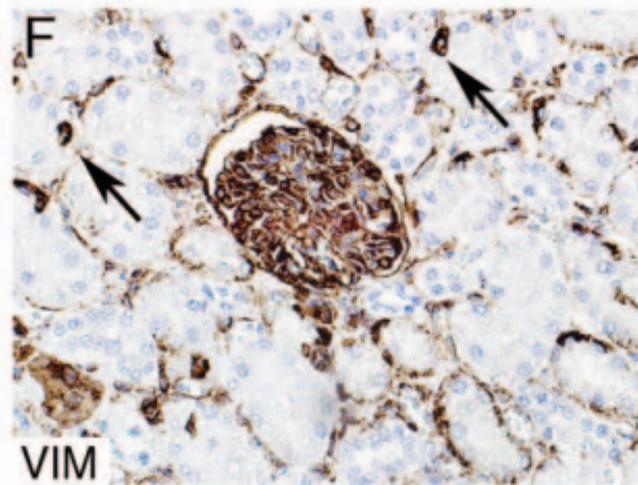
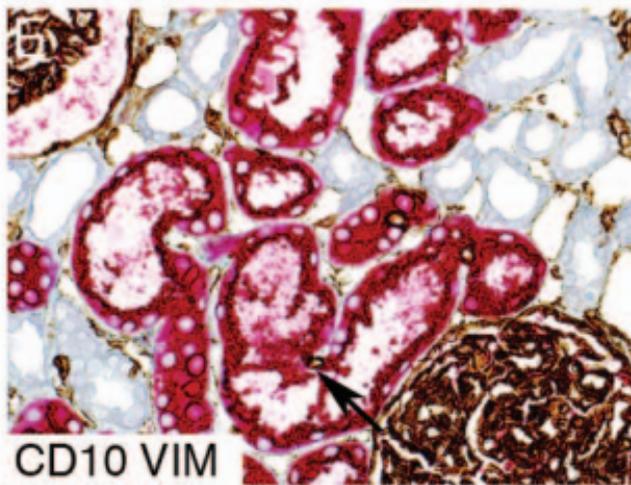
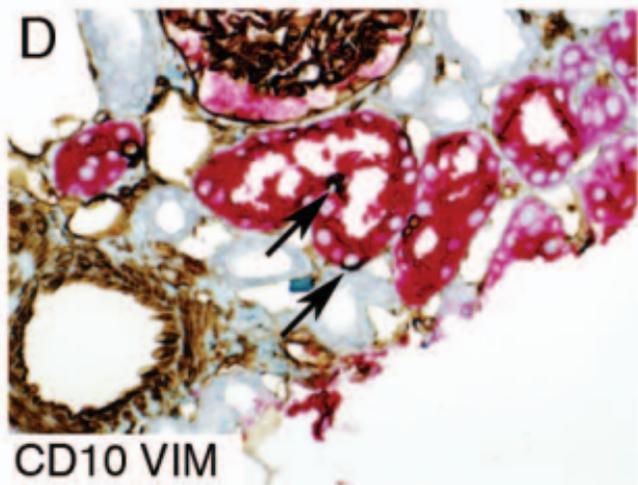
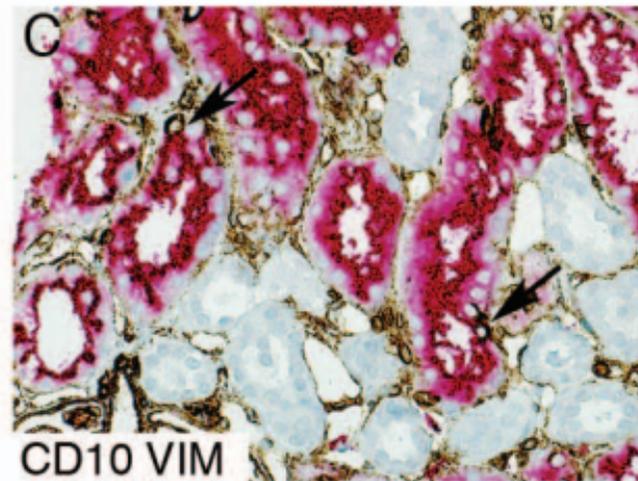
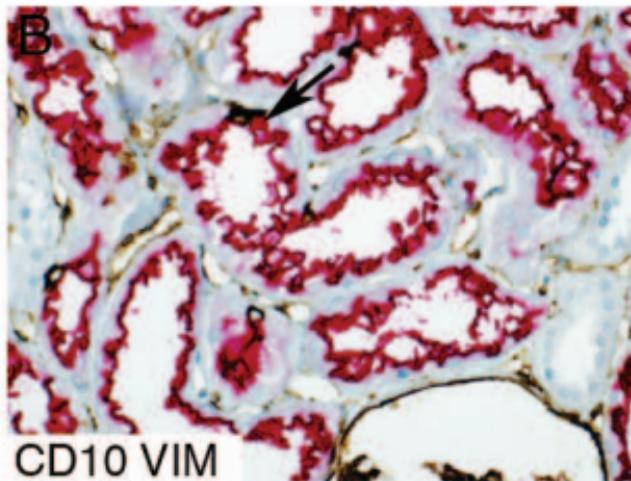
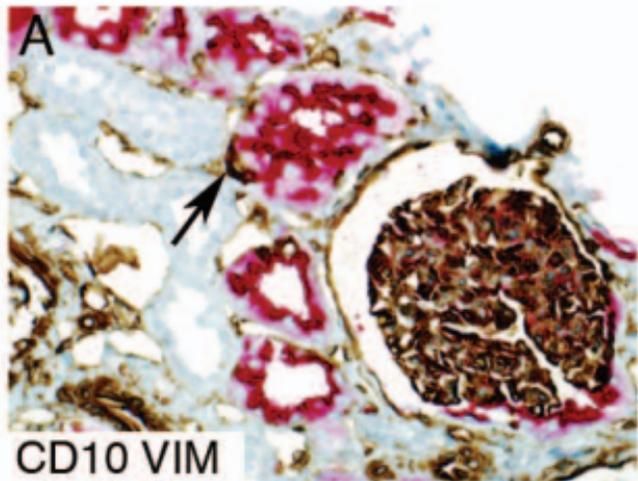
Figure 8. A schematic illustration showing the salient features of scattered cells intermingled among the proximal epithelial cells. The PTRCs are small and flask shaped with no, or less pronounced, brush border. Furthermore, these cells have smaller and darker nuclei than the surrounding cells, indicative of condensed chromatin. The basal compartment of the cells displays a filamentous mat consisting of vimentin and COL7A1, among other structural proteins. Also, the cells have very few mitochondria as compared to the surrounding epithelium. The bulk epithelial cells are on the contrary large, mitochondria rich and display a well-developed brush border.

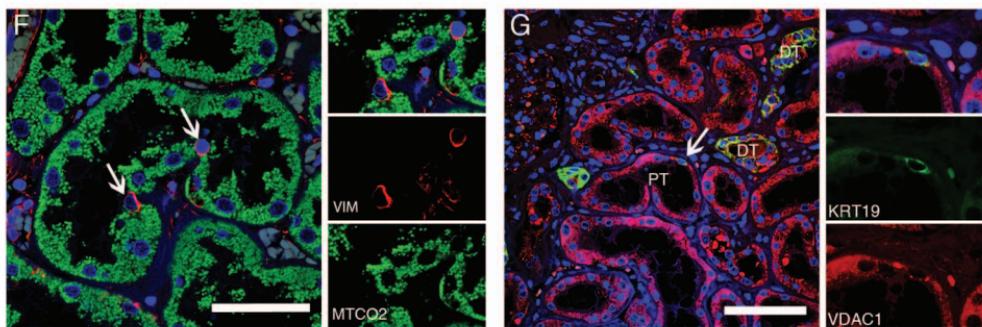
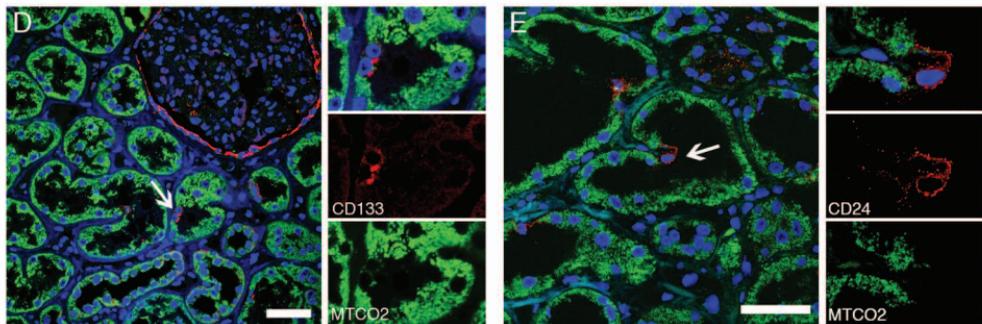
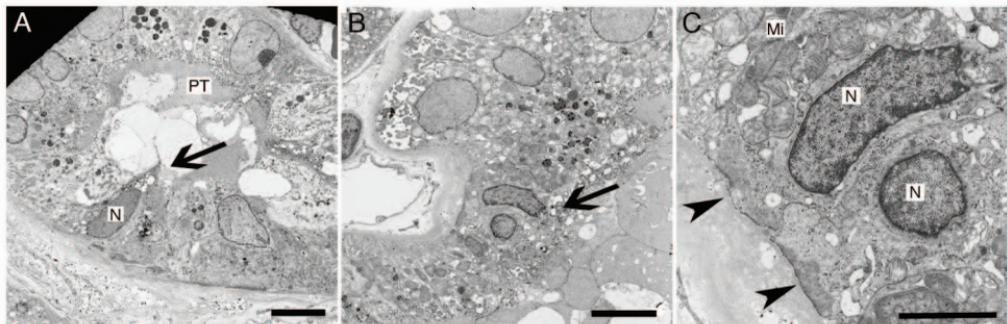
Supplemental Figure 1. Cells with PTRC-markers are resilient to ATN-like insults. (A) Overview of kidney cortex 1.5 h after clamping, H & E staining. (B-E) Staining of renal cortical issue after 72 h of reoxygenation following clamping of kidney. (B) Overview of kidney cortex 72 h after clamping, H & E staining. (C) Immunofluorescent co-staining of CD133 and KRT7. (D) Immunofluorescent co-staining of CD133 and KRT19. (E) Immunofluorescent co-staining of CLDN1 and vimentin. Arrows show cells positive for the progenitor markers still attached to the basal membrane. The right panel (C'-E') shows the same IF slides, stained with PAS Alcian Blue after analysis, to provide for histological analysis. Most cells are necrotic and sloughing from the basal membranes. Scattered single

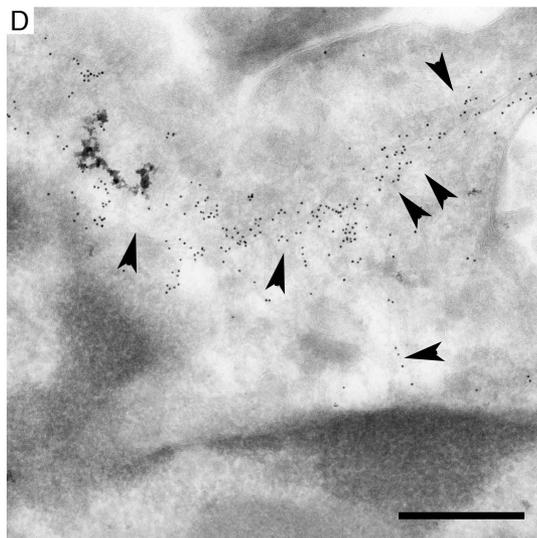
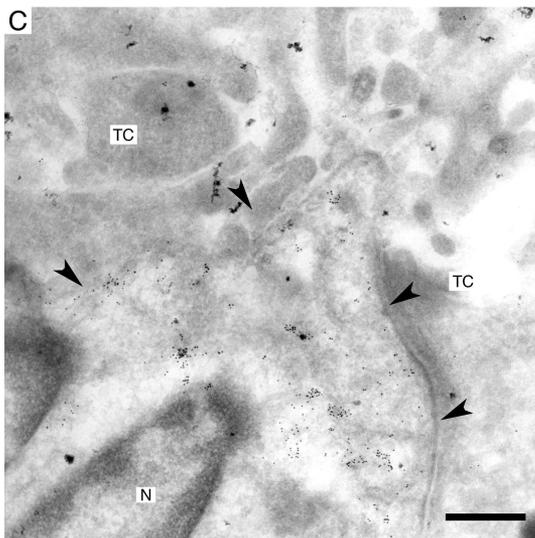
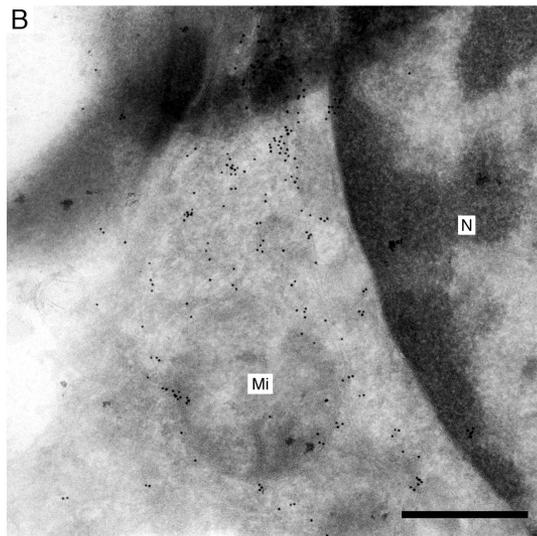
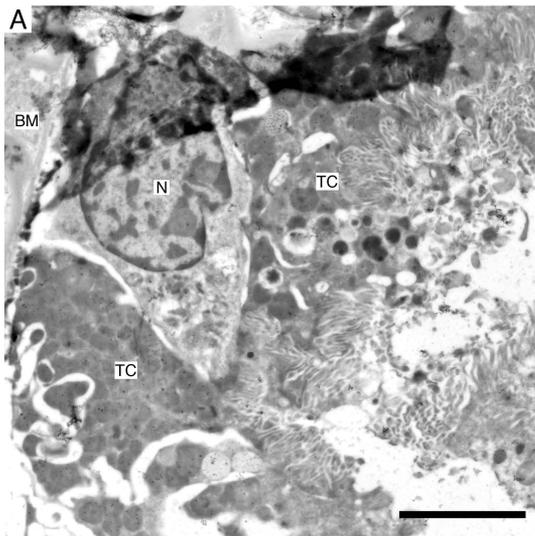
cells are seen still attaching to the tubule, the corresponding IF-images (C-E) show that these attached cells stain for the PTRC-markers. Scale bars: (A) 100 μm , (A'-D) 50 μm .

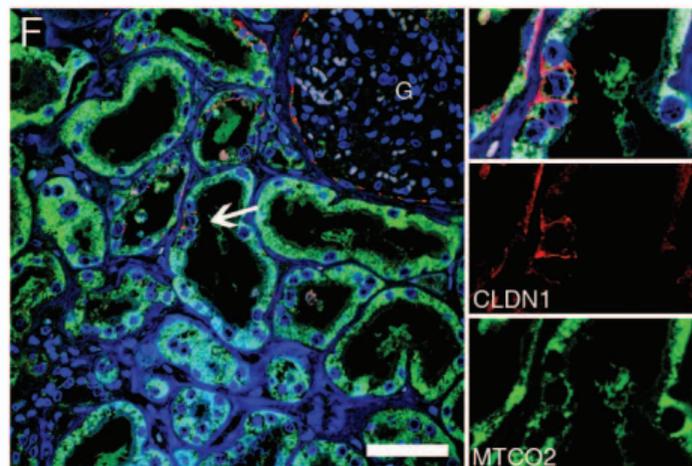
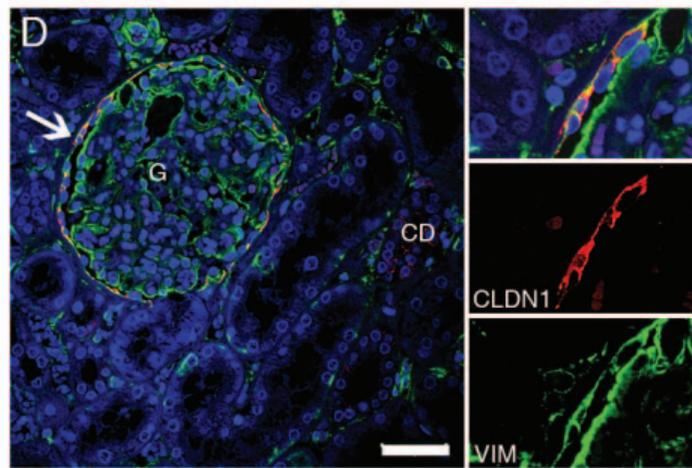
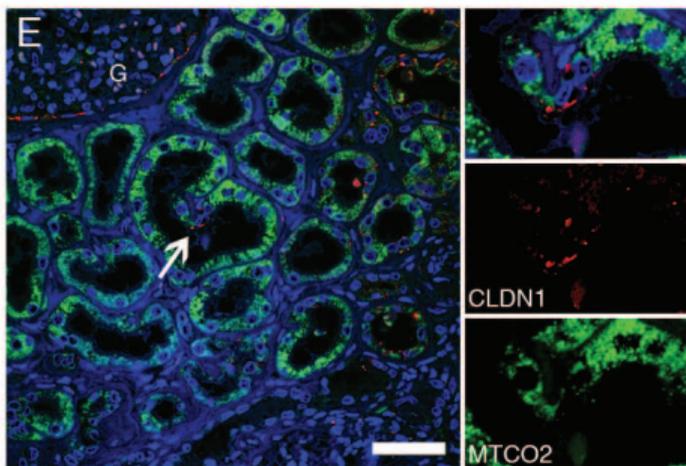
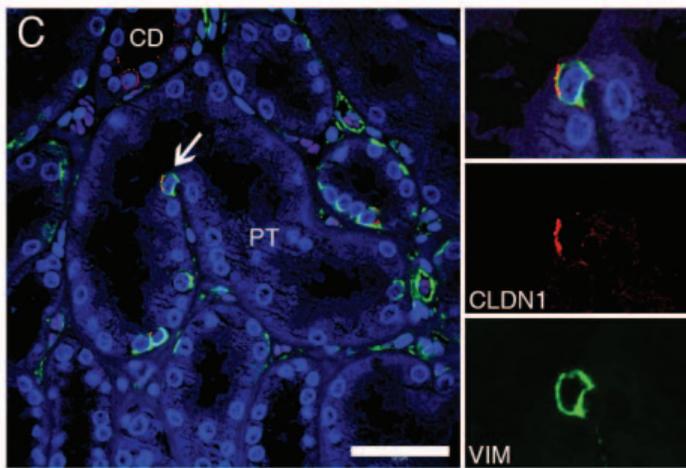
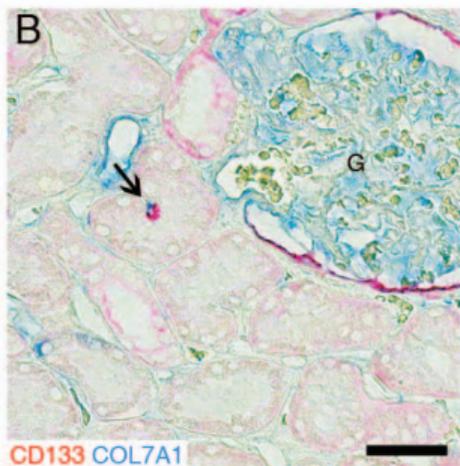
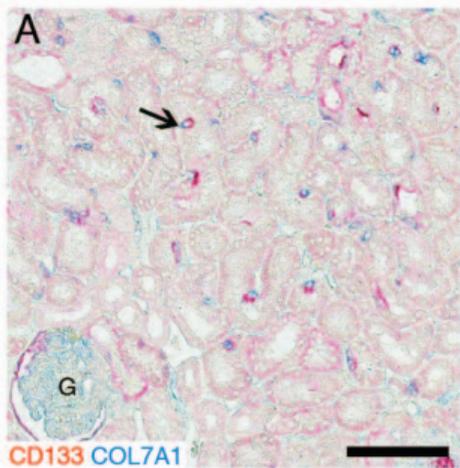
Supplemental Table 1. List of antibodies used for immunohistology.

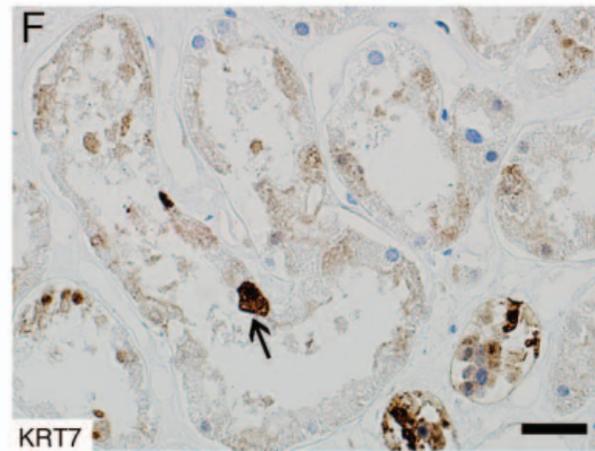
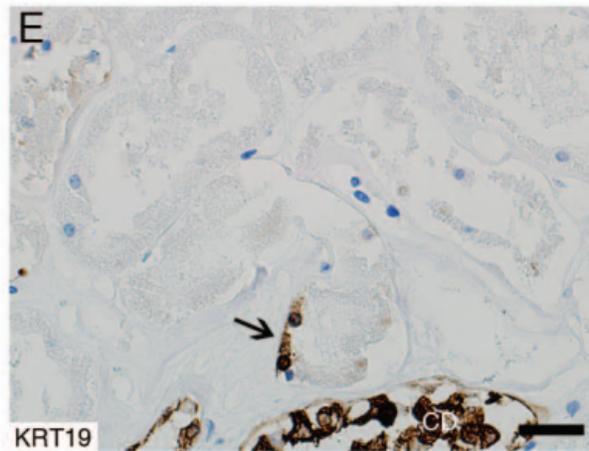
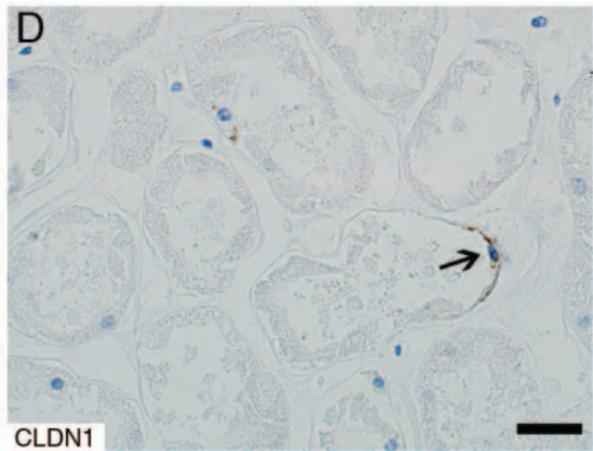
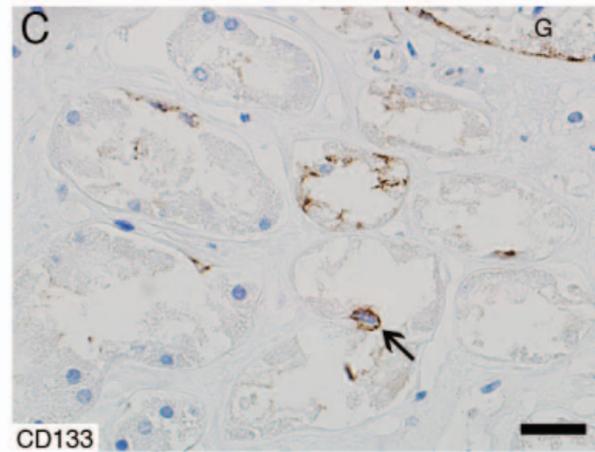
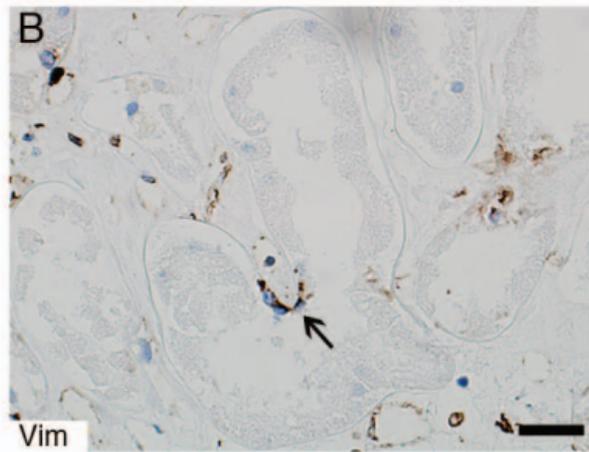
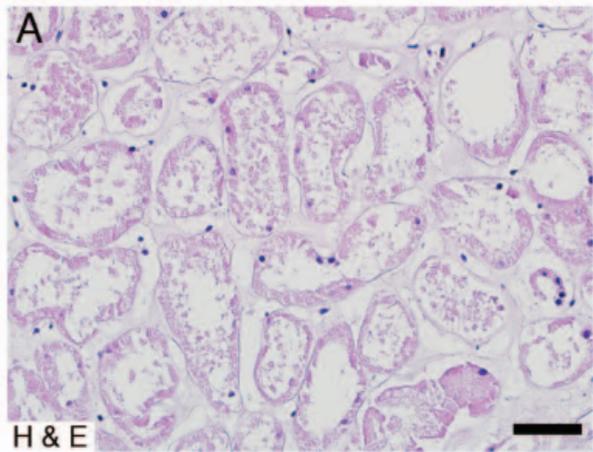




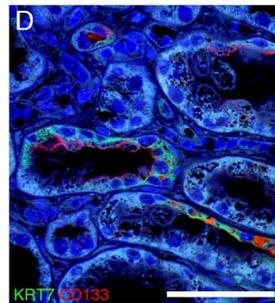
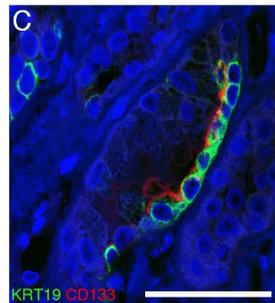
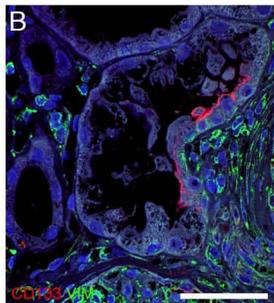
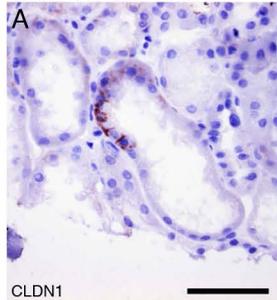




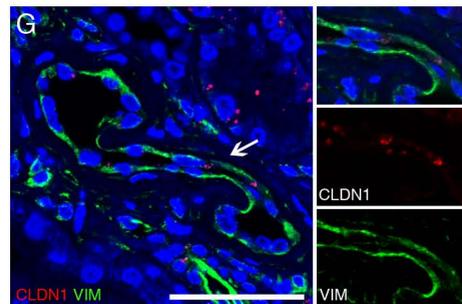
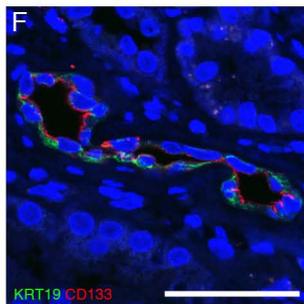
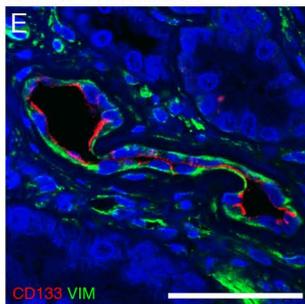




Acute Tubular Necrosis



Glomerulosclerosis



Chronic urethral obstruction

